

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/020464

International filing date: 25 June 2004 (25.06.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/483,352
Filing date: 27 June 2003 (27.06.2003)

Date of receipt at the International Bureau: 30 August 2004 (30.08.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland
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APPLICATION NUMBER: 60/483,352

FILING DATE: June 27, 2003

RELATED PCT APPLICATION NUMBER: PCT/US04/20464

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06/27/03

PTO/SB/16 (10-01)

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Express Mail Label No. EV 335630570 US


60/483352

06/27/03

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<input checked="" type="checkbox"/> Additional inventors are being named on the <u>1</u> separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
METHODS FOR DETECTING NUCLEIC ACID VARIATIONS USING NANOPARTICLES					
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<input checked="" type="checkbox"/> Drawing(s)		Number of Sheets		5	
<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76		<input type="checkbox"/> CD(s), Number			
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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT					
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.				FILING FEE AMOUNT (\$)	
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Respectfully submitted,

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Date 06/27/2003

REGISTRATION NO.

(if appropriate)

Docket Number:

30,480

08321-0148 PX1

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Docket Number	08321-0148 PX1
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METHODS FOR DETECTING NUCLEIC ACID VARIATIONS USING NANOPARTICLES

I. FIELD OF THE INVENTION

[0001] This invention relates to methods for detection of a target molecule in a sample. In particular, the invention relates to the detection and structural analysis of nucleic acid target molecules.

II. BACKGROUND OF THE INVENTION

[0002] Nucleic acid-based detection systems are used to identify a specific nucleic acid sequence within a target molecule. The polymerase chain reaction (PCR) is a technique to specifically amplify known regions of a target nucleic acid molecule by repeated cycles of a chemical reaction that copies a segment of DNA from a template strand. Grattard *et al.*, *J. Clin. Microbiol.* 32: 596-602 (1994) discloses the use of PCR to detect *Enterobacter cloacae* in a nosocomial outbreak.

[0003] PCR based systems use specific primers to detect a target organism. Generally, PCR is used to amplify one or two specific regions of DNA from a target organism. The specificity is determined by the nucleic acid sequence and the length of the primers. Primers are usually on the order of 20-25 nucleic acids in length and specifically bind to the ends of a unique

sequence within a target molecule. Although PCR technology provides substantial sensitivity and selectivity, a major limitation is that nucleic acid-based assays typically only target one species per reaction.

[0004] More recently, methods of identifying known target sequences by probe ligation methods have been reported. See, for example, U.S. Patent No. 4,883,750 to Whiteley *et al.*, Wu, *et al.*, *Genomics* 4:560 (1989); Landegren, *et al.*, *Science* 241:1077 (1988); and Winn-Deen, *et al.*, *Clin. Chem.* 37:1522 (1991). In one approach two probes or probe elements which span a target region of interest are hybridized with the target region. Where the probe elements match adjacent target bases at the confronting ends of the probe elements, the two probe elements can be joined by ligation, *i.e.*, by treatment with ligase. The ligated product is then assayed, evidencing the presence of the target sequence.

[0005] In another approach, the ligated probe elements or ligated products act as a template for an additional pair of complementary probe elements. With continued cycles of denaturation, hybridization, and ligation in the presence of the two complementary pairs of probe elements, the target sequence is amplified exponentially allowing very small amounts of target sequence to be detected and/or amplified. This approach is referred to as ligase chain reaction ("LCR"). See, for example, Jou *et al.*, *Human Mutation* 5:86-93 (1995); and F. Barany, *Proc. Natl. Acad. Sci. USA*, 88:189-93 (1991).

[0006] Several nucleic acid detection methodologies utilize hybridization of labeled oligonucleotides. A novel detection scheme based on nanoparticles was recently described that uses oligonucleotides covalently bound to gold particles. Tatton T, *et al. Science* 289:1757-1760 (2000). Gold nanoparticles tagged with short segments of DNA were used for detection of a

nucleic acid sequence in a sample. Multicolor optical coding for biological assays has been achieved by embedding different-sized quantum dots into polymeric microbeads. Nanopore technology for analysis of nucleic acids converts strings of nucleotides directly into electronic signatures. Jain KK. *Expert Rev Mol Diagn* , 3(2): 153-61 (2003).

[0007] Methods to detect nucleic acid variations are specifically valuable in understanding the genetic make up of disease and pharmacogenetics. Pharmacogenomics is a new approach to drug design, testing and utilization. The premise is that depending on their genetic makeup, individuals respond differently to particular drugs.

[0008] The multifactorial nature of many complex diseases suggests that numerous genes, each with multiple alleles having small to moderate effects, may account for the majority of genetic variations that define risk of chronic diseases on a population basis. Nucleotide variations that are found in at least one percent of the populations are called single nucleotide polymorphisms, or simply SNPs. SNPs occur in roughly one of every 300 bases. Consequently, some several thousands SNPs lie within coding regions of genes. Much of the genetic variation between individual humans that contributes to differences in susceptibility to disease is believed to reflect SNP variations in DNA. Risch and Merikangas *Science* 273: 1516-1517 (1996).

[0009] Some DNA changes cause or strongly contribute to specific diseases. For example, sickle cell anemia is caused solely by the change of an A to a T in the gene encoding the beta chain of hemoglobin. There are many reports of positive association of SNPs with complex diseases such as hypertension (Brand *et al.*, *Hypertension* 32: 198-204. (1998)), or end-stage renal disease (Yu *et al.*, *Hypertension* 31: 906-911 (1998)).

[0010] Although improvements have been made in robotic manipulation of nucleic acid reagents through technologies such as DNA chip array, LCR, and nanobiotechnology, and although particular processes such as amplification of nucleic acid molecules and sequencing are well developed, there still exists a need for techniques and devices for rapid and efficient analysis and detection of nucleic acid molecules and for the integration of systems that assist in reduction of the number of manipulations required to perform biological assays for detection of nucleic acid variations in a sample. This invention addresses these and many other needs in the detection of nucleic acid molecules.

[0011] The detection method of the present invention incorporates nanoparticle-based detection systems and achieves several orders of magnitude higher sensitivity and selectivity than the analogous molecular fluorescence-based approach. Oligonucleotides covalently attached to nanoparticles provide both improved hybridization discrimination as well as increased sensitivity on microarrays. Additionally, the nanoparticle-based methodology of the invention advantageously uses Raman spectrum that offers potentially greater flexibility, a larger pool of available and non-overlapping probes, and higher multiplexing capabilities than conventional nucleic acid detection approaches.

III. SUMMARY OF THE INVENTION

[0012] The invention, as disclosed and described herein, provides methods and kits for detecting nucleic acid variations in a target nucleic acid molecule.

[0013] In one aspect, the invention provides a method of identifying nucleic acid variations in a sample comprising: a) providing a sample potentially containing one or more target polynucleotides; b) providing one or more oligonucleotide probe sets, each set

characterized by (i) a first oligonucleotide probe, having a target-specific portion and a first barcode, and (ii) a second oligonucleotide probe, having a second target-specific portion and a second barcode, wherein the first target-specific portion in the first oligonucleotide probe and the second target-specific portion in the second oligonucleotide probe in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target molecule, but have a mismatch which interferes with such ligation when hybridized to any other polynucleotide present in the sample; c) providing a ligase; d) blending the sample, the plurality of oligonucleotide probe sets, and the ligase to form a mixture; e) subjecting the mixture to one or more ligase detection reaction cycles comprising a denaturation treatment and a hybridization treatment, wherein the oligonucleotide probe sets hybridize at adjacent positions to form a ligated product containing the first barcode, the target-specific portions connected together, and the second barcode; f) providing a solid support with one or more surface-bound probes on an array, wherein the surface-bound probes are complementary to the first barcode; g) contacting the ligated product of step e) with the solid support under conditions effective for hybridization of the first barcode with the surface-bound probes; h) providing a third barcode carrying one or more detectable labels and a nanoparticle attached therein, wherein the third barcode is complementary to the second barcode; i) and detecting the presence of the detectable labels on the ligated product captured on the solid support at particular sites, thereby indicating the presence of one or more target molecules in the sample.

[0014] In another embodiment, the invention provides a method of identifying nucleic acid sequence variations in a sample comprising: a) providing a sample potentially containing one or more target polynucleotide with a plurality of nucleotides variations; b) providing at least three oligonucleotide probe sets, each set characterized by (i) a first oligonucleotide probe,

having a first target-specific portion and a first barcode, (ii) a second oligonucleotide probe, having a second target-specific portion and a second barcode; and (iii) a third oligonucleotide probe, having a third target-specific portion and a third barcode, wherein the first target-specific portion in the first oligonucleotide probe in a particular set is suitable for ligation with the second target-specific portion in the second oligonucleotide probe, or the third target-specific portion in the third oligonucleotide probe, but have a mismatch which interferes with such ligation when hybridized to any other polynucleotide present in the sample; c) providing a ligase; d) blending the sample, the plurality of oligonucleotide probe sets, and the ligase to form a mixture; e) subjecting the mixture to one or more ligase detection reaction cycles comprising a denaturation treatment, wherein any hybridized oligonucleotides are separated from the target polynucleotide, and a hybridization treatment, wherein the oligonucleotide probe sets hybridize at adjacent positions to form at least two ligated products, the first ligated product containing the first barcode, the first target-specific portion ligated to the second target specific portion and the second barcode, and the second ligated product containing the first-barcode, the first target-specific portion ligated to the third target-specific portion and the third barcode; f) providing a solid support with a plurality of surface-bound probes on an array, wherein the surface-bound probes are complementary to the first barcode; g) contacting the first and the second ligated product of step (e) with the solid support under conditions effective for hybridization of the first barcode with the surface-bound probes; h) providing a fourth barcode carrying one or more detectable labels and a nanoparticle attached therein, wherein the fourth barcode is complementary to the second barcode; i) providing a fifth barcode carrying one or more detectable labels and a nanoparticle attached therein, wherein the fifth barcode is complementary to the third barcode; j) and detecting the presence of the detectable labels on the first ligated

product, the second ligated product, or both on the solid support a particular site, thereby indicating the presence of one or more target polynucleotides in the sample. The nucleotide to be detected being located, for example, in the first target-specific portion immediately adjacent to the junction of the first and the second target-specific portions, or the first and third target-specific portions of the oligonucleotide probe sets.

[0015] In one embodiment, the detectable label comprises various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, disperse dyes, or a combination thereof. In a preferred embodiment, the detectable label is a dye. In a more preferred embodiment, the label is one or more dyes with different surface-enhanced Raman spectra signatures.

[0016] The dye comprises, for example, cyanine dye (including CyA, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, and Cy7.5.3; R110 (5&6-carboxyrhodamine-110); R6G (6-carboxyrhodamine-6G); TAMRA (N,N,N',N'-tetramethyl-6-carboxyrhodamine); ROX (6-carboxy-X-rhodamine); FAM (6-carboxyfluorescein); JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein); HEX (6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein); TET (6-carboxy-2',4,7,7'-tetrachlorofluorescein); ZOE (5-carboxy-2',4',5',7'-tetrachlorofluorescein), R6, NAN, Texas Red, Rhodamine Red, Alexa dyes, or a combination thereof, among others.

[0017] In one embodiment, the detection method of the invention detects single nucleotide polymorphisms. In another embodiment, the detection method detects variations in one or multiple allele differences at single or multiple nucleotide positions. The nucleic acid sequence variations comprise insertions, deletions, microsatellite repeats, translocations, mutations, or a combination thereof.

[0018] In another embodiment, the target specific portion of the probes is from about 15 to about 30 nucleotides long and have substantially the same melting temperature as the target polynucleotide so that it hybridizes to the target polynucleotides under similar hybridization conditions. For example, the target-specific portion of the probe has a hybridization temperature of from about 40 °C to about 85 °C. Preferably the hybridization temperature is from about 60 °C to about 70 °C.

[0019] In yet another embodiment, the denaturation treatment prior to or after the ligase reaction is completed at a temperature of about 70 °C to about 105. The duration of the denaturation treatment is in the range of about 30 seconds to about 5 minutes, for example, 50 seconds, 100 seconds, 2 minutes, 2.5 minutes, 3 minutes, and 4 minutes. It is intended herein that the ranges recited also include all those specific numerical amounts between the recited ranges.

[0020] In one embodiment, the ligase enzyme comprises *Thermus aquaticus* ligase, *Thermus thermophilus* ligase, *E. coli* ligase, T4 DNA ligase, *Thermus* sp. AK16 ligase, *Aquifex aeolicus* ligase, *Thermotoga* *maritima* ligase, and *Pyrococcus* ligase, among others.

[0021] In another embodiment, the target nucleic acid is amplified by a polymerase chain reaction prior to ligation.

[0022] In yet another embodiment, the ligated product is amplified by a polymerase chain reaction after ligation.

[0023] In yet another embodiment, the ligated product is treated chemically or enzymatically after ligation to remove unligated oligonucleotide probes. This step can be carried out, for example, by treatment with an exonuclease.

[0024] In another aspect, the present invention provides a diagnostic test kit for detection of nucleic acid variations in a sample. The diagnostic test kit includes, for example, one or more of the following: templates, buffers, LCR probes and reagents, PCR primers and reagents, hybridization reagents, other chemical agents, nucleotides, probe arrays, enzymes, nanoparticles, detection agents, barcodes, control materials, devices, and the like, among others.

[0025] In one embodiment, at least two nanoparticle-attached barcodes are provided in separate containers.

[0026] In another embodiment, the nanoparticles and barcodes each are separately contained and conjugation of the nanoparticle with the barcode is carried out prior to the assay.

[0027] In yet another embodiment, the nanoparticles, the barcodes attached to the nanoparticles, or both are functionalized prior to attachment of nanoparticles.

[0028] In another embodiment, the test kit additionally contains the probe array or microarray on a substrate.

[0029] These and other aspects and embodiments of the invention are disclosed in detail herein.

IV. BRIEF DESCRIPTION OF THE FIGURES

[0030] Figure 1. Shows a schematic for LCR detection of a nucleic acid variation in a target molecule. FIG. 1A shows a target nucleic acid, having a site to be interrogated shown by a clear circle. The target nucleic acid is prepared using complementary strand from PCR product or an unamplified genomic DNA. Fig 1B shows a schematic representation of two LCR probes used to detect the nucleic acid variation in the target molecule. Each of the LCR probes has a complementary target-specific portion (I b and II a) and a barcode (I a and II b) at their respective 5' and 3' ends. One barcode binds a complementary surface-bound probe on an array, and the second barcode binds a complementary solution-phase barcode containing an attached nanoparticle.

[0031] Figure 2. Shows a schematic representation of the ligation between the LCR probes and the target nucleic acid molecule. Fig. 2A shows two LCR probes (I and II) containing a target-specific portion (I b and II a) which recognizes the complementary target sequences. The target-specific portions I b and II a being contiguous so that the first oligonucleotide probe and the second oligonucleotide probe abut one another in 5' phosphate-3' hydroxyl relationship. Fig. 2B shows that a ligase reaction has covalently fused or ligated the two oligonucleotide probes into a ligated product containing two target-specific portions (I b and II a) and two barcodes (I a and II b).

[0032] Figure 3. Shows a schematic representation of the interaction between the solution phase ligated product and a nanoparticle-attached barcode with the solid phase probe array. Fig. 3A shows the ligated product containing two target-specific portions (I b and II a) and two barcodes (I a and II b) in solution phase. Fig. 3B shows the gold nanoparticle-attached barcode III in solution phase. Fig. 3C shows the solid phase probe array. The probe IV is

attached to the solid surface by a linker moiety. The solid surface is functionalized by an amino group moiety prior to the attachment to probe IV.

[0033] Figure 4. Shows a schematic representation of the capture of the ligated product on the solid surface and detection by the gold nanoparticle. The first barcode (Ia) attached to the ligated product is hybridized with the probe (IV) on the array. The second barcode (II b) on the ligated product is hybridized with the barcode (III) attached to the gold (AU) nanoparticle.

[0034] Figure 5. Shows a schematic representation for the capture of two alleles on one spot on the array. Three LCR probes are provided. The first LCR probe having the first target-specific portion (I b) and a first barcode (I a). The second LCR probe having the second target-specific portion (II a) and the second barcode (II b). The third LCR probe having the third target-specific portion (III a) and a third barcode (III b). The first barcode in each LCR probe is capable of binding with the probe array (VI). Two gold (AU) nanoparticle-attached barcodes (barcodes IV and V) are provided. Barcode IV is complementary to the second barcode (II b) and barcode V is complementary to the third barcode (III b) Each nanoparticle is attached to dye (dye 1 and dye 2) having a different surface-enhanced Raman spectra signature.

V. DETAILED DESCRIPTION OF THE INVENTION

[0035] Methods and kits for automated detection and identification of a target nucleic acid molecule are described herein. The invention provides capability of detection of multiple target molecules concomitantly and rapidly. To detect the presence and/or concentration of a target molecule, the invention as described herein employs nanoparticles to create a highly sensitive, and specific surface enhanced spectral Raman-based detection system.

[0036] The detection method of the invention is highly sensitive and is capable of detecting minutes amounts of a target agent (*i.e.*, as little as 100, 10, or 1 molecule in the sample), and is highly reliable in that the interaction is specific for each target agent.

[0037] The methods and probe compositions of the invention are useful in a variety of prognostic, diagnostic, and detection applications. These applications include, by way of example and not limitation, detection, identification and/or quantification of genetic abnormalities or genetic diseases in mammalian, detection of cancer, detection of bacterial infections (*i.e.*, tuberculosis, Lyme disease, *H. pylori*, *Escherichia coli* infections, Legionella infections, Mycoplasma infections, Salmonella infections, among others); detection of viral infections (*i.e.*, human immunodeficiency virus, hepatitis viruses, herpes viruses, cytomegalovirus, and Epstein-Barr virus, among others); detection of sexually transmitted diseases; detection of inherited disorders (*i.e.*, cystic fibrosis, Duchenne muscular dystrophy, phenylketonuria, sickle cell anemia), detection of cancer (*i.e.*, genes associated with the development of cancer); forensic science, determination of a genetic relationship, such as paternity or species identification, determination of potential donors of organs or tissues, the

identification of a sequence (gene/gene mutation) and the determination of an expression level (abundance) of genes, among others.

1. Definitions

[0038] The definitions used in this application are for illustrative purposes and do not limit the scope of the invention.

[0039] As used herein, “nucleic acid molecule” or polynucleotide” includes cDNA, RNA, DNA/RNA hybrid, anti-sense RNA, ribozyme, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified to contain non-natural or derivitized, synthetic, or semi-synthetic nucleotide bases. Also, included within the scope of the invention are alterations of a wild type or synthetic gene, including, but not limited to deletion, insertion, substitution of one or more nucleotides, or fusion to other polynucleotides.

[0040] As used herein, “oligonucleotide probe” includes DNA, RNA, DNA-RNA hybrid, antisense RNA, cDNA, genomic DNA, mRNA, ribozyme, a natural, synthetic, or recombinant nucleic acid molecule, aptamers and/or peptide-nucleic acid hybrid, among others. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art.

[0041] As used herein, “polymerase-mediated reaction” refers to a biochemical reaction in which a template molecule or a population of template molecules is periodically and repeatedly copied to create a complementary template molecule(s), thereby increasing the number of the complementary template molecules over time. Generally, a “polymerase-

mediated reaction" includes primer molecules that serve to initiate synthesis of the complementary molecule, a polymerase enzyme that catalyzes the synthesis, and monomeric molecules that make up the product. In each cycle of a "polymerase-mediated reaction" not every template will necessarily be copied, and each complementary molecule whose synthesis is initiated in a cycle will not necessarily be completed.

[0042] As used herein, "template molecule" refers to a molecule that can serve as a template for the synthesis of a complementary molecule. Most often, a "template molecule" is a polymeric molecule. The "template molecule" is a nucleic acid molecule, *i.e.* DNA, RNA, DNA-RNA hybrid, antisense RNA, cDNA, genomic DNA, a natural, synthetic, recombinant nucleic acid molecule, or a combination thereof. In the context of PCR or ligation a "template molecule" may represent a fragment or fraction of the nucleic acids added to the reaction. Specifically, a "template" refers to the sequence between and including the two primers.

[0043] As used herein "microarray" refers to an arrangement of distinct polynucleotides arrayed on a substrate, *i.e.*, paper, nylon or any other type of membrane, filter, chip, glass slide, or any other suitable solid support.

[0044] As used herein, "target molecule" refers to a molecule whose structure, presence and/or abundance is being detected. Most often, a "target molecule" is a polymeric molecule. In preferred embodiments, a "target molecule" is a polynucleotide molecule including DNA, RNA, DNA-RNA hybrid, antisense RNA, cDNA, genomic DNA, mRNA, ribozyme, a natural, synthetic, or recombinant nucleic acid molecule, and peptide-nucleic acid hybrid. A target molecule can be derived from any of a number of sources, including animals, plants, bacteria, fungi, viruses, and the like. In certain embodiments, the target molecule is a polynucleotide

whose sequence structure, presence or absence can be used for certain medical, forensic, or biological detection purposes.

[0045] As used herein, "sample," includes any sample containing a target molecule that can be detected by composition and methods of the invention. Samples may be obtained from any source including animals, plants, fungi, bacteria, and viruses, among others. Animal samples are obtained, for example from tissue biopsy, blood, hair, buccal scrapes, plasma, serum, skin, ascites, plural effusion, thoracentesis fluid, spinal fluid, lymph fluid, bone marrow, respiratory, intestinal fluid, genital fluid, stool, urine, sputum, tears, saliva, tumors, organs, tissues, samples of *in vitro* cell culture constituents, fetal cells, placenta cells or amniotic cells and/or fluid.

[0046] As used herein, the term "detectable label" includes molecules that are attached to a nucleic acid molecule of the invention and either alone or in combination with a binding partner assist in detection of a hybridization product.

[0047] As used herein, the term "barcode" refers to short oligonucleotides with no complementary nucleic acid sequence to a target nucleic acid sequence that may be present in a sample. Thus, the barcode does not hybridize with any target nucleic acid sequence that is contained in the sample. Preferably, the sequence of the barcode is selected such that it has no complement in the genome of the species from which the sample is derived from.

[0048] The invention, as described herein, provides a novel detection methods and probes involving solid-phase capture and identification of nucleic acid sequence variants

combining two different technologies (*i.e.*, ligase chain reaction-LCR and nanoparticles-based detection).

[0049] In one embodiment, the detection method of the invention involves solution-phase ligation of at least two templated (*i.e.*, complementary to the target sequence) oligonucleotide probes having different target-specific portions and different barcodes at their respective 5' and 3' ends. One barcode binds a complementary surface-bound probe on an array, and the second barcode binds a complementary solution-phase barcode. The barcodes are short oligonucleotides with no complementary nucleic acid sequence to a target nucleic acid sequence that may be present in a sample. Thus, the barcodes do not hybridize with any target nucleic acid sequence that is contained in the sample.

[0050] The complementary solution-phase barcode carries a nanoparticle and one or more detectable labels attached thereto. The nanoparticle is preferably attached at the end of the barcode. The target-specific portions of the oligonucleotide probes are able to ligate together when they hybridize adjacent to one another on a corresponding target polynucleotide, but have a mismatch which interferes with such ligation when hybridized to any other polynucleotides present in the sample.

[0051] In another embodiment, the detection method of the invention involves solution-phase ligation of at least three templated oligonucleotide probes, each having a target-specific portion and a barcode at their respective 5' and 3' ends. One barcode binds a complementary surface-bound probe on an array, and the second barcode binds a complementary solution-phase barcode. This method allows the capture of at least two ligated products, one representative of a normal target polynucleotide, and the other representative of a mutant target polynucleotides on

one site on the array. The capture of the ligated products or a mutant target polynucleotide by the probe on the array results in one or more barcodes becoming available for hybridization to the solution-phase barcode carrying one or more detectable labels that are placed adjacent to a nanoparticle.

[0052] In another embodiment, the detectable label is one or more detectable dyes, each detectable dye having a different surface-enhanced Raman spectra (SERS signatures). Raman spectroscopy can then be used for the detection of any number of different dyes as detectable labels. For example, one or more separate dyes are used with different surface-enhanced Raman spectra signatures, so that one spot on an array can be used to genotype both the normal and variant sequence (*i.e.*, homozygous normal (N/N), heterozygous (N/M), or homozygous mutant (M/M)).

2. Nanoparticles

[0053] The methods and detection kits of the invention exploit recent laboratory breakthroughs in nanotechnology through the use of nanoparticles. Nanoparticles have a very small diameter, are very sensitive to changes in their external environment, and may or may not be gated with charged molecules. The small size and high surface-to-volume ratio makes nanoparticles extremely sensitive to changes in their external environment. Nanoparticles are capable of detecting and identifying femtogram (10^{-15} g) quantities of a target molecule, within one minute. When used in a diagnostic capacity, nanoparticles preferably detect the target molecules directly without any additional reagents. The response of nanoparticles is fast and selective for the particular target molecule under examination.

[0054] Nanoparticles include, for example, nanowires (aka nanorods, *see*, for example, U.S. Patent No. 6,190,634 issued to Lieber *et al.*), nanowire arrays, quantumdots (aka nanodots), nanotubes (*see*, for example, Ebbesen *et al.*, *Nature* 358: 220-222 (1992), and Stephan *et al. Science*: 266, 1683-1685(1994)), each of which is incorporated herein by reference in its entirety.

[0055] The sensitivity and selectivity of the nanoparticle for a specific application is designed through manipulations of several factors including, for example, materials that nanoparticles are made of, coating materials, doping materials, and diameter of the nanoparticles, among others. In one embodiment, the sensitivity of a nanoparticle is designed through a manipulation of doping and particle diameter. The size of the nanoparticles is preferably from about 5 nm to about 150 nm (mean diameter), more preferably from about 5 nm to about 50 nm, most preferably from about 10 nm to about 30 nm.

[0056] Nanoparticles are made of , for example, metal (*i.e.*, gold, silver, copper and platinum), semiconductor (*i.e.*, CdSe, CdS, and CdS or CdSe coated with ZnS) and magnetic (*i.e.*, ferromagnetite) colloidal materials. Other nanoparticles are made of, for example, ZnO, TiO₂, AgI, AgBr, HgI₂, PbS, PbSe, ZnTe, and CdTe, among others.

[0057] Methods of making metal, semiconductor and magnetic nanoparticles are well-known in the art. *See, i.e.*, Schmid, G. (ed.) *Clusters and Colloids* (VCH, Weinheim, 1994); Hayat, M. A. (ed.) *Colloidal Gold: Principles, Methods, and Applications* (Academic Press, San Diego, 1991); Ahmadi, *et al.*, *Science*, 272, 1924 (1996); and Henglein, *et al.*, *J. Phys. Chem.*, 99, 14129 (1995). Suitable nanoparticles are also commercially available from, *i.e.*, Ted Pella, Inc. (gold), Amersham Corporation (gold) and Nanoprobes, Inc. (gold).

[0058] In a number of other embodiments of the invention, the detectable change is created by labeling the barcodes, nanoparticles, or both with detectable labels (*i.e.*, fluorescent molecules and/or dyes) that produce detectable changes upon hybridization of the barcode – attached nanoparticle with the barcode attached to a ligated product. Methods of detecting nucleic acids based on observing a color change with the naked eye are efficient, simple and economical. Methods of labeling oligonucleotides with fluorescent or dye molecules are well known in the art.

[0059] Suitable fluorescent molecules and dyes are well known in the art and include, for example, fluoresceins, aminoacridine, TRIT, and NBD (4-chloro-7-nitrobenzo-2-oxa-1-diazole) umbelliferone, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride, phycoerythrin, luminal, luciferase, luciferin, aequorin, CyA, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, and Cy7.5.3, R110 (5&6-carboxyrhodamine-110), R6G (6-carboxyrhodamine-6G); TAMRA, N,N,N' (N'-tetramethyl-6-carboxyrhodamine); ROX (6-carboxy-X-rhodamine; FAM, 6-carboxyfluorescein); JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein); HEX (6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein); TET (6-carboxy-2',4,7,7'-tetrachlorofluorescein); ZOE (5-carboxy-2',4',5',7'-tetrachlorofluorescein); R6, NAN, Texas Red, Rhodamine Red, or a combination thereof, among others.

[0060] In a preferred embodiment of the invention, the gold are placed in the vicinity of a Raman-active dye in order to increase the strength of the unique Raman spectra exhibited by the dye. Hybridization of a barcode-attached gold nanoparticles with oligonucleotides attached to a ligated product results in an unique Raman spectra, which is indicative of the presence of a target sequence. This method offers potentially greater flexibility, a larger pool of available and non-

overlapping probes, and higher multiplexing capabilities than conventional fluorescence-based detection approaches.

[0061] Multiplexed detection of oligonucleotide targets has been performed with gold nanoparticle probes labeled with Raman-active dyes. *See, i.e., Yunwei, et al., Science, 297: 1536 (2002), incorporated herein by reference in its entirety.* Gold nanoparticles act as a surface-enhanced Raman scattering promoter for the dye-labeled particles that have been captured by target molecules on a chip in a microarray format. facilitate the formation of a silver coating that acts as a surface-enhanced Raman scattering promoter for the dye-labeled particles that have been captured by target molecules on a chip in a microarray format. Before silver enhancing, the nanoparticle probes are almost invisible to the naked eye, and no Raman scattering signal is detectable. After silver enhancing, the silver particles can grow around the dye (*i.e., Cy3*)-labeled gold nanoparticle probes, leading to large Raman scattering enhancements.

[0062] Dyes other than Cy3 can be used to create a large number of probes with distinct and measurable SERS signals for multiplexed detection. The number of available Raman dyes is much greater than the number of available and discernable fluorescent dyes. For example, a Raman dye can be either fluorescent or nonfluorescent, but a minor chemical modification of a dye molecule can lead to a new dye with a different Raman spectrum even though the two dyes exhibit virtually indistinguishable fluorescence spectra. *See, for example, Graham, et al., Chem. Int. Ed. 39: 1061 (2000), Kneipp et al. Chem. Rev. 99: 2957 (1999), each of which is incorporated herein by reference in its entirety.*

[0063] The nanoparticles, barcodes or both are functionalized in order to attach the nanoparticle to the barcode. Such functionalization methods are known in the art. For instance,

oligonucleotides functionalized with alkanethiols at their 3'-termini or 5'-termini readily attach to gold nanoparticles. Other functional groups for attaching oligonucleotides to solid surfaces include phosphorothioate groups (*see, i.e.*, U.S. Patent No. 5,472,881 for the binding of oligonucleotide-phosphorothioates to gold surfaces), substituted alkylsiloxanes (*see*, Burwell, *Chemical Technology*, 4: 370-377 (1974) and Matteucci and Caruthers, *J. Am. Chem. Soc.*, 103: 3185-3191 (1981) for binding of oligonucleotides to silica and glass surfaces, each of which is incorporated herein by reference in its entirety. Oligonucleotides terminated with a 5' thionucleoside or a 3' thionucleoside may also be used for attaching oligonucleotides to solid surfaces.

3. *Sample/Target*

[0064] The invention provides methods of detecting a nucleic acid target molecule in a sample. Any type of nucleic acid target may be detected. Examples of nucleic acids that can be detected by the methods of the invention include genes (*i.e.*, a gene associated with a particular disease), viral, bacterial, and fungal nucleic acid molecules, among others. The nucleic acid molecules to be detected are isolated by known methods, or are detected directly in cells, tissue samples, biological fluids (*i.e.*, saliva, urine, blood, and serum), solutions containing PCR products, solutions containing large excesses of polynucleotides or high molecular weight DNA, and other samples, as also known in the art. *See, i.e.*, Sambrook *et al.*, *supra*. If a target nucleic acid is present in small amounts, it may be amplified by methods known in the art.

[0065] Samples used in the methods of this invention can be obtained from any source that potentially contains a target molecule. Such sources include animals, plants, soil, water, fungi, bacteria, and viruses, among others. Animal samples are obtained, for example from

tissue biopsy, blood, hair, buccal scrapes, plasma, serum, skin, ascites, plural effusion, thoracentesis fluid, spinal fluid, lymph fluid, bone marrow, respiratory, intestinal fluid, genital fluid, stool, urine, sputum, tears, saliva, tumors, organs, tissues, samples of *in vitro* cell culture constituents, fetal cells, placenta cells or amniotic cells and/or fluid.

[0066] Methods for the preparation of samples can be found in a multitude of sources, including Sambrook *et al.*, *supra*. Any such method can be used in the present invention as an upstream processing. Typically, these methods involve cell lysis, followed by purification of nucleic acids by methods such as phenol/chloroform extraction, electrophoresis, and/or chromatography. Often, such methods include a step wherein the nucleic acids are precipitated, *i.e.* with ethanol, and resuspended in an appropriate buffer for addition to a PCR reagent, LCR reagent or other nucleic acid amplification reagents.

[0067] The choice of the template used within a sample depends on the particular application used. Such applications include diagnostic procedures, wherein the presence or absence of a particular nucleic acid provides information regarding, for example, the existence or state of a disease. In other applications, nucleic acids are amplified for use in a downstream application, such as for use as a probe, or for sequencing, *i.e.* to determine the precise sequence of a particular genetic locus in one or more individual samples.

[0068] In certain embodiments, a plurality of template molecules from one or more samples is used. For example, a single nucleic acid from a multitude of samples may be amplified to screen for the presence or absence of a particular nucleic acid molecule. In other applications, a plurality of nucleic acids may be amplified from a single sample or an individual, thereby allowing the assessment and simultaneous screening for a multitude of disease markers

in the individual. Any of the above applications can be easily accomplished using the detection method and kits of the invention described herein.

[0069] The present invention further relates to polynucleotide target molecules that hybridize to the herein-described oligonucleotide probe sequences. The term “hybridization under stringent conditions” according to the present invention is used as described by Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press 1.101-1.104, 1989. Preferably, a stringent hybridization according to the present invention is given when after washing for an hour with 1% SSC and 0.1 % SDC at 50°C, preferably at 55° C, more preferably at 62° C, most preferably at 68°C, a positive hybridization signal is still observed. The oligonucleotide probes of the invention include polynucleotide sequences that have homology and complementarity to a target sequence.

4. Amplification Strategies

4.1. LCR

[0070] The amplification reaction, according to the invention described herein, includes a ligase chain reaction (LCR) or a variation thereof. Ligase chain reaction (LCR) is a mechanism for target amplification. In LCR, two contiguous oligonucleotide probes are joined by DNA ligase upon perfect hybridization to a polynucleotide target. The ligated products are equivalent to either the target or its complement. By repeated cycles of hybridization and ligation, amplification of the target sequence is achieved.

[0071] In one embodiment of the invention, LCR is performed with two primary probes (first and second probes) and two secondary (third and fourth) probes are employed in excess. The first probe hybridizes to a first portion of a target strand and the second probe hybridizes to a

second portion of a target strand, the first and second portions being contiguous so that the primary probes abut one another in 5' phosphate-3' hydroxyl relationship so that a ligase can covalently fuse or ligate the two probe. In addition, a third (secondary) probe can hybridize to a portion of the first probe and a fourth (secondary) probe can hybridize to a portion of the second probe in a similar abutting fashion.

[0072] If the target is initially double stranded, the secondary probes will also hybridize to the target complement in the first instance. Once the fused strand of primary probes is separated from the target strand, it will hybridize with the third and fourth probes which can be ligated to form a complementary, secondary fused product. LCR is described by, for example, Backman, *et al.* in EP-A-320 308; and Barany, *Proc. Natl Acad Sci USA* 88:189-193 (1991), each of which is incorporated herein by reference in its entirety.

[0073] The hybridization step, discriminates between nucleotide sequences based on a distinguishing nucleotide at the ligation junctions. The difference between the target nucleotide sequences can be, for example, a single nucleic acid base difference, a nucleic acid deletion, a nucleic acid insertion, or rearrangement. Such sequence differences involving more than one base can also be detected.

[0074] Also encompassed within the scope of the invention is a ligase chain reaction (LCR) wherein at least one probe is modified so that when hybridized with its complementary probe, the resulting duplex is not "blunt-ended" (*i.e.* ligatable) with respect to the partner complementary probe duplexes. After hybridizing to the target, the modified ends are "corrected" in a target dependent fashion to render the adjacent probes ligatable. For example, the probes have recesses relative to the point of ligation which create a gap when hybridized to the target.

The gap is then filled in a target dependent manner to render the probes ligatable. A "modified end" has, for example, a blocking moiety, additional base residues on a group (*i.e.* the 5' phosphate or the 3' hydroxyl) which, under ordinary LCR conditions, obligatorily participates in the ligase catalyzed fusion, or omitted bases to create a "gap" between one probe terminus and the next probe terminus.

[0075] In the "gap" embodiment, modified ends are created by eliminating from one or more of the probes a short sequence of bases, thereby leaving a recess or gap between the 5' end of one probe and the 3' end of the other probe when they are both hybridized to the target (or target complement, or polynucleotide generated therefrom). In order for LCR to amplify the target, the gaps between the probes must be filled in (*i.e.*, the modification must be "corrected"). In a first version, this can be done using a polymerase or a reverse transcriptase and an excess of deoxynucleotide triphosphates which are complementary to the target strand opposite the gap. Alternatively, this can be done by supplying a fifth probe complementary to the target and a sixth probe complementary to the fifth probe. *See*, for example, U.S. Patent No. 5,427,930 issued to Birkenmeyer, *et al.*, incorporated herein by reference in its entirety.

[0076] LCR oligonucleotide probes of defined sequences are used for a variety of purposes in the practice of the invention. Methods of making oligonucleotides of a predetermined sequence are well-known. *See, i.e.*, Sambrook *et al. supra*. F. Eckstein (ed.) Oligonucleotides and Analogues, 1st Ed. (Oxford University Press, New York, 1991). Also encompassed within the scope of the invention are LCR oligonucleotide probes having removed repetitive sequences therefrom.

5. Solid Surface

[0077] The invention described herein relies on the use of a solid phase substrate for the capture of the target nucleic acid. Any substrate can be used which allows observation of the detectable change upon capture of the target nucleic acid on the substrate. Suitable substrates include transparent solid surfaces (*i.e.*, glass, quartz, plastics and other polymers), opaque solid surface (*i.e.*, white solid surfaces, such as TLC silica plates, filter paper, glass fiber filters, cellulose nitrate membranes, nylon membranes), and conducting solid surfaces (*i.e.*, indium-tin-oxide (ITO)). The substrate can be any shape or thickness.

[0078] In one embodiment, multiple oligonucleotide probes are attached to the substrate. The oligonucleotides can be attached to the substrates as described in, for example, Chrisey *et al.*, Nucleic Acids Res., 24: 3040-3047 (1996); and Graves *et al.*, Patent Publication No. 20030099930, each of which is incorporated herein by reference in its entirety.

[0079] According to one embodiment of the invention, the oligonucleotide probe attached to the substrate has a sequence complementary to the first barcode contained within a ligated product. A ligated product having a first barcode, the target specific portions connected together and a second barcode is contacted with the oligonucleotide probe on the substrate under conditions effective to allow hybridization of the first barcode with the oligonucleotide probe on the substrate.

[0080] Figure 4 shows a schematic representation of the capture of the ligated product on the solid surface and detection by the gold nanoparticle. The first barcode (Ia) attached to the ligated product is hybridized with the probe (IV) on the array. The second barcode (II b) on the ligated product is hybridized with the barcode (III) attached to the gold (AU) nanoparticle. In this manner the ligated product becomes bound to the substrate. Any unbound ligated product or

probe is preferably washed from the substrate before adding nanoparticle-oligonucleotide conjugates.

[0081] In another embodiment, the oligonucleotide probe bound to the substrate is contacted with two or more ligated products. One ligated product contains a first barcode, the target-specific portions connected together, and the second barcode. Another ligated product contains a first barcode, the target specific portions connected together and the third barcode. The first barcode has a sequence that is complementary to the oligonucleotide probe attached to the substrate. Upon contact of the ligated products with the substrate under conditions effective to allow hybridization, one or both of the ligated products will attach to the substrate *via* hybridization of the first barcode with the oligonucleotide probe. This method is used to capture, for example, two alleles on one spot on the array. Figure 5 shows a schematic representation for the capture of two alleles on one spot on the array. In this Figure three LCR probes are used. The first LCR probe having the first target-specific portion (I b) and a first barcode (I a). The second LCR probe having the second target-specific portion (II a) and the second barcode (II b). The third LCR probe having the third target-specific portion (III a) and a third barcode (III b). The first barcode in each LCR probe is capable of binding with the probe array (VI). Two gold (AU) nanoparticle-attached barcodes (barcodes IV and V) are provided. Barcode IV is complementary to the second barcode (II b) and barcode V is complementary to the third barcode (III b) Each nanoparticle is attached to dye (dye 1 and dye 2) having a different surface-enhanced Raman spectra signature.

6. *Test Kits*

[0082] Also encompassed within the scope of the invention are diagnostic test kits for detecting nucleic acids.

[0083] Generally, kits contain one or more of the following: (1) one or more containers containing the reagents and probes as described herein; (2) instructions for practicing the methods described herein; (3) one or more assay component; and (4) packaging materials. The reagents and probes described herein are packaged to include many if not all of the necessary components for performing the detection of nucleic acid molecules. For example, kits can include any of templates, buffers, LCR probes and reagents, PCR primers and reagents, hybridization reagents, other chemical agents, nucleotides, probe array, enzymes, nanoparticles, detection agents, barcodes, reagents for silver staining, control materials, devices, or the like, among others.

[0084] Other items which may be provided as part of the kit include a solid surface (for visualizing hybridization) such as a TLC silica plate, microporous materials, syringes, pipettes, cuvettes, containers, and a thermocycler (for controlling hybridization and de-hybridization temperatures). Reagents for functionalizing the nucleotides or nanoparticles may also be included in the kit.

[0085] In the case of LCR amplification, the kit comprises, for example, multiple disposable reaction/detection units and one or more containers holding in suitable buffer(s), a DNA ligase, NAD, and at least two probes specific for amplifying a predetermined target nucleic acid by the ligase chain reaction. In some variations of LCR, there may also be included a

polymerase or other enzyme for "correction" of probes to improve sensitivity by reducing non-specific background ligation.

[0086] In one embodiment, the kit has the barcodes and nanoparticles in separate containers, and the barcodes would have to be attached to the nanoparticles prior to performing an assay to detect a nucleic acid variation. The barcodes and/or the nanoparticles may be functionalized so that the barcodes can be attached to the nanoparticles. Alternatively, the barcodes and/or nanoparticles may be provided in the kit without functional groups, in which case they must be functionalized prior to performing the assay.

[0087] In another embodiment, the kit comprises at least one container. The container holds metallic or semiconductor nanoparticles having barcodes attached thereto. The barcodes have fluorescent molecules attached to their ends.

[0088] In yet another embodiment, the kit comprises a substrate, the substrate having attached thereto a probe or a universal probe. The nanoparticles have barcodes attached thereto which have a sequence complementary to one or more additional barcodes.

[0089] In another embodiment, the kit comprises a container holding one type of nanoparticles having barcodes attached thereto and one or more types of oligonucleotide probes. Each of the oligonucleotide probes has a sequence comprising at least two portions. The first portion is a barcode complementary and the second portion is target-specific complementary.

[0090] Detection can be facilitated by coupling a barcode, a probe, or a nanoparticle to a detectable agent. Examples of detectable agents include, but are not limited, to various enzymes,

prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, disperse dyes, gold particles, or a combination thereof.

[0091] Examples of suitable detectable agents, as disclosed above, include suitable enzymes, *i.e.*, horseradish peroxidase, alkaline phosphatase, betagalactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include, but are not limited to, streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include, but are not limited to, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes, but is not limited to, luminol; examples of bioluminescent materials include, but are not limited to luciferase, luciferin, and aequorin; and examples of suitable radioactive material include, but are not limited to ^{125}I , ^{35}S , ^{14}C , ^3H , $^{99\text{M}}\text{Tc}$, or ^{52}Mg .

[0092] In a preferred embodiment, the detectable agent is one or more dyes attached to a nanoparticle barcode. In a preferred embodiment the dye is CyA, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, and Cy7.5.3; R110 (5&6-carboxyrhodamine-110); R6G (6-carboxyrhodamine-6G); TAMRA (N,N,N',N'-tetramethyl-6-carboxyrhodamine); ROX (6-carboxy-X-rhodamine); FAM (6-carboxyfluorescein); JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein); HEX (6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein); TET (6-carboxy-2',4,7,7'-tetrachlorofluorescein); and ZOE (5-carboxy-2',4',5',7'-tetrachlorofluorescein); R6, NAN, Texas Red, Rhodamine Red, or Alexa dyes, among others.

[0093] All references discussed herein are incorporated by reference. One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present

invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

What is claimed is:

1. A method of identifying a target nucleic acid sequence variation comprising;
 - a. providing a sample potentially containing one or more target polynucleotide;
 - b. providing one or more oligonucleotide probe sets, each set characterized by (i) a first oligonucleotide probe, having a first target-specific portion and a first barcode, and (ii) a second oligonucleotide probe, having a second target-specific portion and a second barcode, wherein the first oligonucleotide probe and the second oligonucleotide probe in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target polynucleotide;
 - c. providing a ligase,
 - d. blending the sample, the one or more oligonucleotide probe sets, and the ligase to form a mixture;
 - e. subjecting the mixture to one or more ligase detection reaction cycles comprising a hybridization treatment, and a denaturation treatment, wherein the oligonucleotide probe sets hybridize at adjacent positions to form a ligated product containing the first barcode, the target-specific portions connected together, and the second barcode;
 - f. providing a solid support with one or more surface-bound probes on an array, wherein the surface-bound probes are complementary to the first barcode;

- g. contacting the ligated product of step (e) with the solid support under conditions effective for hybridization of the first barcode with the surface-bound probes;
 - h. providing a third barcode carrying one or more detectable labels and a nanoparticle attached therein, wherein the third barcode is complementary to the second barcode; and
 - i. detecting the presence of the detectable labels on the ligated product captured on the solid support at particular sites, thereby detecting the nucleic acid sequence variation in the sample.
2. The method of Claim 1, wherein the target nucleic acid sequence variation is a single nucleotide polymorphism.
 3. The method of Claim 1, wherein the surface-bound probes capture a normal target polynucleotide, a mutant target polynucleotide, or both.
 4. The method of Claim 3, wherein the nanoparticle is attached at a 5' end or a 3' end of the third barcode.
 5. The method of Claim 1, wherein the detectable labels comprise one or more dyes.
 6. The method of Claim 5, wherein the one or more dyes have different surface-enhanced Raman spectra signatures
 7. The method of Claim 5, wherein one or more dyes comprise cyanine dye, R110, R6, TAMRA, ROX, FAM, JOE, ZOE, TET, HEX, NAN, Texas Red, Rhodamine Red, Alexa dyes, or a combination thereof.

8. The method of Claim 7, wherein the cyanine dye comprises CYA, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, and Cy7.5.3.

9. The method of Claim 1, wherein the mutant target polynucleotide differs from the target polynucleotide in the sample at one or more single nucleotide positions.

10. The method of Claim 1, wherein the nucleic acid sequence variations comprises multiple allele differences at a single nucleotide position.

11. The method of Claim 1, wherein the nucleic acid sequence variations comprise multiple allele differences at two or more nucleotide positions.

12. The method of Claim 1, wherein the nucleic acid sequence variations comprise multiple allele differences at nucleotide positions in multiple target polynucleotides.

13. A method of Claim 1, wherein the target-specific portions of the oligonucleotide probe sets have substantially the same melting temperature so that they hybridize to the target polynucleotides under similar hybridization conditions.

14. The method of Claim 1, wherein the nucleic acid sequence variations comprise insertions, deletions, microsatellite repeats, translocations, mutations, or a combination thereof.

15. The method of Claim 1, wherein the denaturation treatment is at a temperature of about 70 °C to about 105

16. The method of Claim 1, wherein the target-specific portions of the oligonucleotide probes each have a hybridization temperature of from about 40 °C to about 85 °C.

17. The method of Claim 16, wherein the hybridization temperature is from about 60 °C to about 70 °C.
18. The method of Claim 1, wherein the denaturation and the hybridization is from about 30 seconds to about 5 minutes long.
19. The method of Claim 1, wherein step (e) is repeated for about 2 to about 50 cycles.
20. The method of Claim 1, wherein step (e) takes about 1 to 250 minutes.
21. The method of Claim 1, wherein the ligase is selected from the group consisting of *Thermus aquaticus* ligase, *Thermus thermophilus* ligase, *E. coli* ligase, T4 DNA ligase, *Thermus* sp. AK16 ligase, *Aquifex aeolicus* ligase, *Thermotoga maritima* ligase, and *Pyrococcus* ligase.
22. The method of Claim 1, wherein the target-specific portions of the oligonucleotide probes are from about 15 to about 30 nucleotides long.
23. The method of Claim 1 further comprising, amplifying the target polynucleotides in the sample prior to the ligase.
24. The method of Claim 23, wherein the amplifying is carried out by subjecting the sample to a polymerase-based amplifying procedure.
25. The method of Claim 1, wherein the solid support is made from a material selected from the group consisting of plastic, ceramic, metal, resin, gel, glass, silicon, and composites thereof.

26. The method of Claim 1, further comprising treating the ligated product chemically or enzymatically after step (e) to remove unligated oligonucleotide probes.

27. The method of Claim 25, wherein the treating step is carried out with an exonuclease.

28. The method of Claim 1, wherein the target polynucleotide is a genomic DNA.

29. The method of Claim 1, wherein the ligated product is amplified with random primers and DNA polymerase after ligation.

30. A method of identifying nucleic acid sequence variations in a sample comprising;

a. providing a sample potentially containing one or more target polynucleotide with at least one nucleotide variation;

b. providing one or more oligonucleotide probe sets, each set characterized by (i) a first oligonucleotide probe, having a first target-specific portion and a first barcode, (ii) a second oligonucleotide probe, having a second target-specific portion and a second barcode; and (iii) a third oligonucleotide probe, having a third target-specific portion and a third barcode, wherein the first target-specific portion in the first oligonucleotide probe in a particular set is suitable for ligation with the second target-specific portion in the second oligonucleotide probe, or the third target-specific portion in the third oligonucleotide probe;

c. providing a ligase;

- d. blending the sample, the plurality of oligonucleotide probe sets, and the ligase to form a mixture;
- e. subjecting the mixture to one or more ligase detection reaction cycles comprising a hybridization treatment and a denaturation treatment, wherein the oligonucleotide probe sets hybridize at adjacent positions to form at least two ligated product, the first ligated product containing the first barcode, the first target-specific portion connected with the second target-specific portion, and the second barcode, the second ligated product containing the first barcode, the first target-specific portion connected with the third target-specific portion, and the third barcode;
- f. providing a solid support with a plurality of surface-bound probes on an array, wherein the surface-bound probes are complementary to the first barcode;
- g. contacting the first and the second ligated product of step (e) with the solid support under conditions effective for hybridization of the first barcode with the surface-bound probes;
- h. providing a fourth barcode carrying one or more detectable labels and a nanoparticle attached therein, wherein the fourth barcode is complementary to the second barcode;
- i. providing a fifth barcode carrying one or more detectable labels and a nanoparticle attached therein, wherein the fifth barcode is complementary to the third barcode; and

j. detecting the presence of the detectable labels on the first ligated product, the second ligated product, or both on the solid support at a particular site, thereby indicating the presence of one or more nucleic acid variation in a sample.

31. The method of Claim 30, wherein the second and the third oligonucleotide probes capture allelic variants of a target polynucleotide.

32. The method of Claim 30, wherein the detectable labels carried by the fourth barcode comprises dye 1 and the detectable labels carried by the fifth barcode comprises dye 2.

33. The method of Claim 33, wherein the dye 1 and the dye 2 have different surface-enhanced Raman spectra signatures.

34. The method of Claim 1, wherein the nucleic acid variations comprises a single nucleotide polymorphism.

35. The method of Claim 34, wherein the dye 1 and the dye 2 comprising a cyanine dye, R110, R6, TAMRA, ROX, FAM, JOE, ZOE, TET, HEX, NAN, Texas Red, Rhodamine Red, or Alexa dye, among others.

36. The method of Claim 36, wherein the cyanine dye comprises CYA, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, and Cy7.5.

37. A diagnostic test kit for detection of nucleic acid variations in a sample comprising (a) a plurality of oligonucleotide probe sets, each set characterized by (i) a first oligonucleotide probe, having a target-specific portion and a first barcode, and (ii) a second oligonucleotide probe, having a target-specific portion and a second barcode, wherein the first

oligonucleotide probe and the second oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target polynucleotide, but have a mismatch which interferes with such ligation when hybridized to any other polynucleotide present in the sample; (b) ligase reagents; (c) a third barcode carrying one or more detectable labels and a nanoparticle attached therein, wherein the third barcode is complementary to the second barcode.

38. The diagnostic test kit of Claim 38, wherein the third barcode comprises at least two different barcodes.

39. The diagnostic test kit of Claim 38, wherein the nanoparticle and the third barcode are in separate containers, and the third barcode is attached to the nanoparticles prior to performing an assay.

40. The diagnostic test kit of Claim 38, wherein the nanoparticle, the third barcode, or both are functionalized prior to attachment of the nanoparticle.

41. The diagnostic test kit of Claim 38 further containing a substrate, the substrate having attached thereto a probe that hybridizes to the first barcode. |

42. The diagnostic test kit of Claim 42, wherein the probe is a DNA microarray.

METHODS FOR DETECTING NUCLEIC ACID VARIATIONS USING NANOPARTICLES

ABSTRACT

The invention provides methods and diagnostic test kits for detecting a target nucleic acid sequence variations in a sample. In particular, a plurality of oligonucleotide probe sets is provided. Each set has a target specific portion and a barcode. The target specific portions of the probes are suitable for ligation together when hybridized adjacent to one another on a corresponding target polynucleotide. The ligated product contains a barcode that binds to a probe attached to a substrate and hence facilitate the capture of the ligated product on the solid support. Detection is carried out with a gold nanoparticle-attached barcode that hybridizes with the barcode on the ligated product.

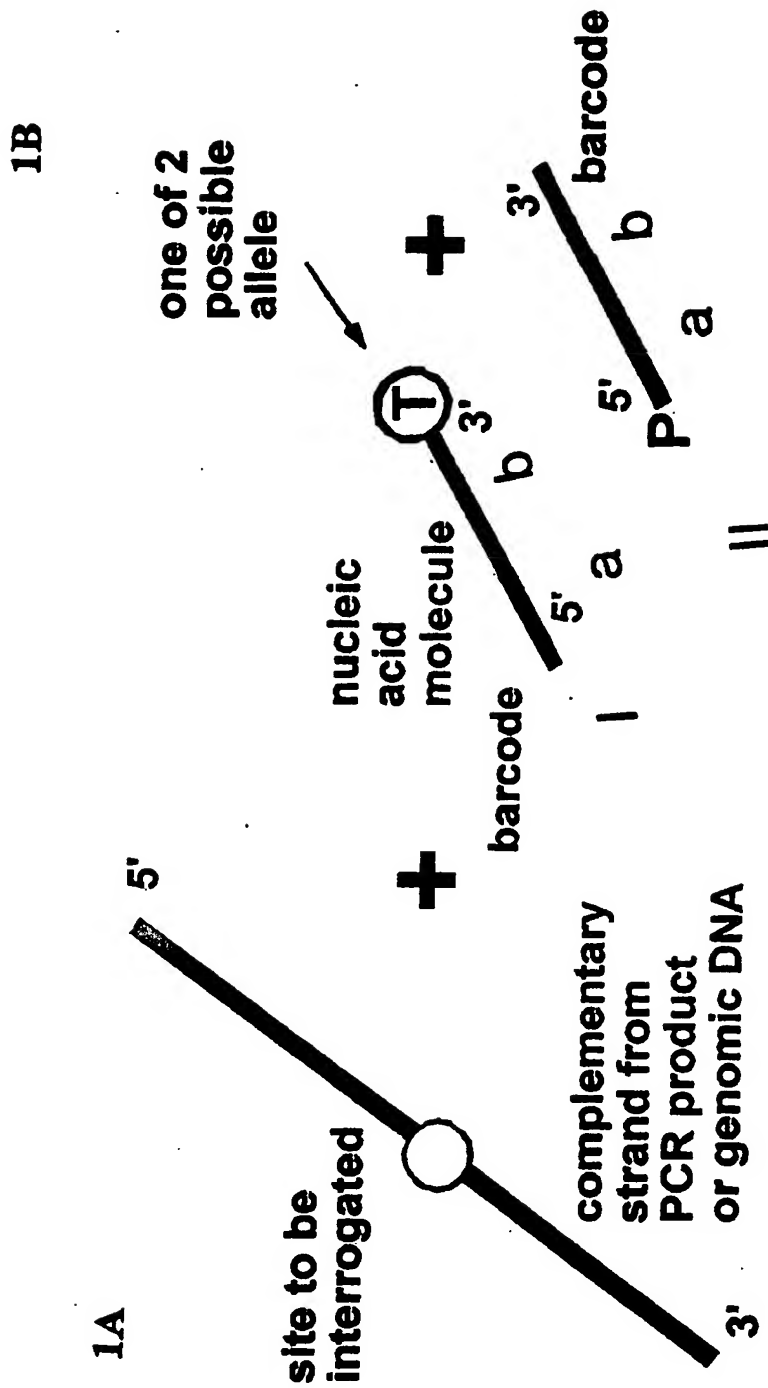


FIG. 1/5

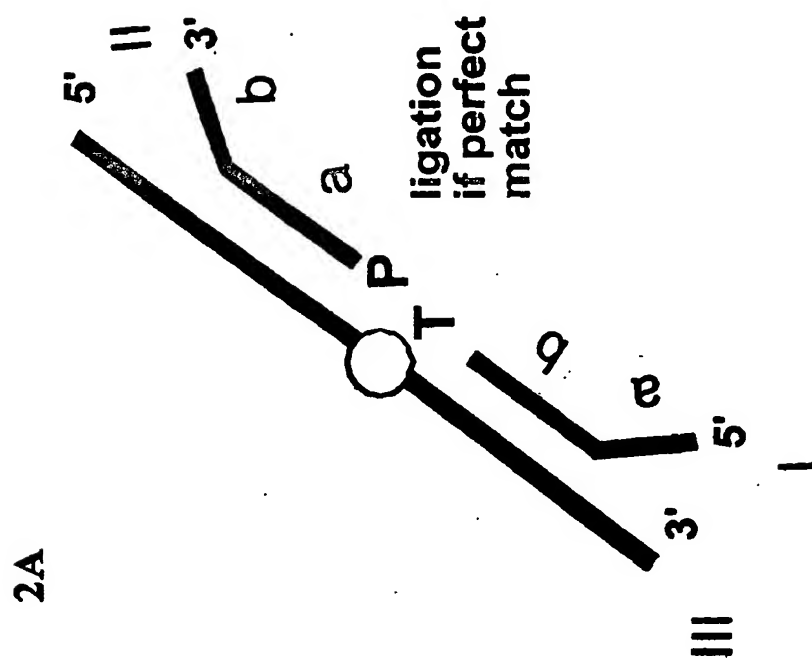
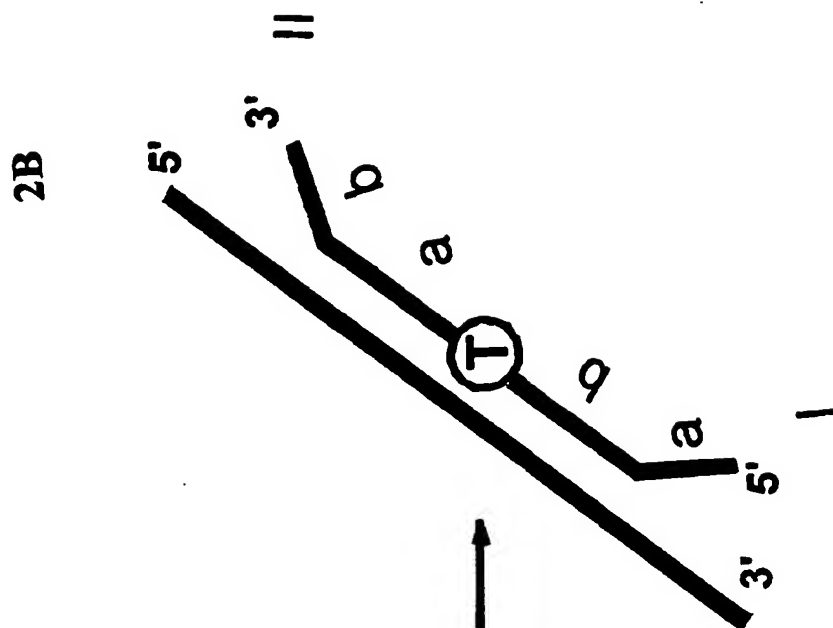


FIG. 2/5

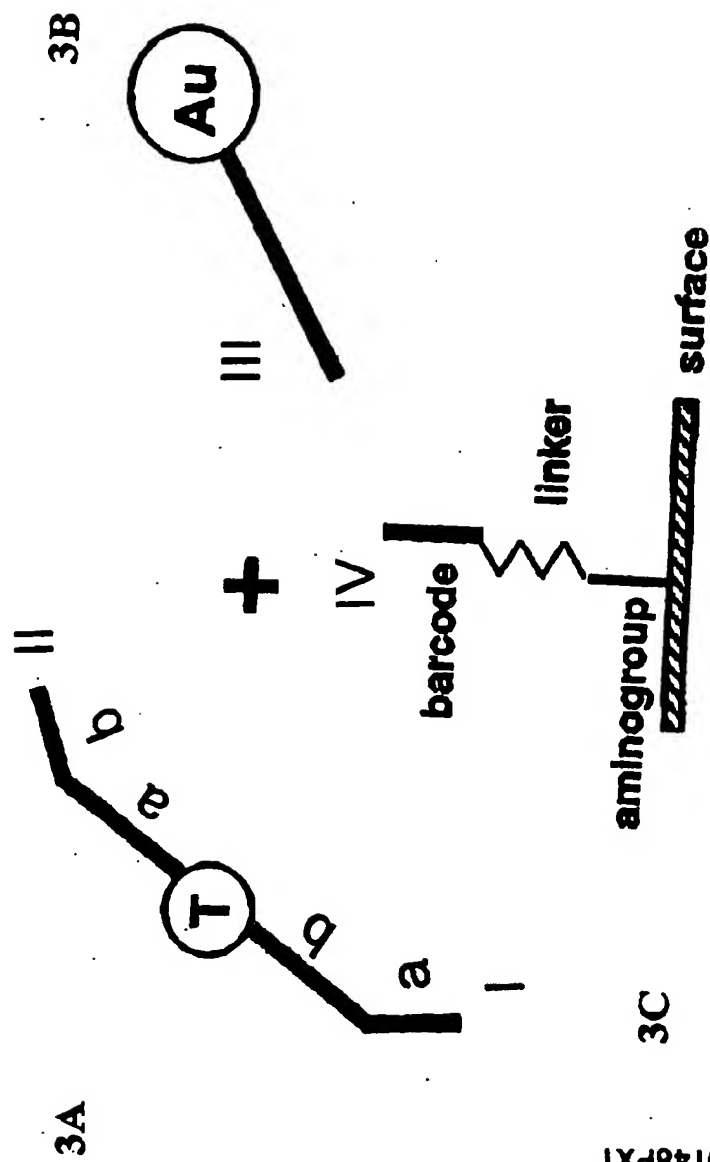


FIG. 3/5

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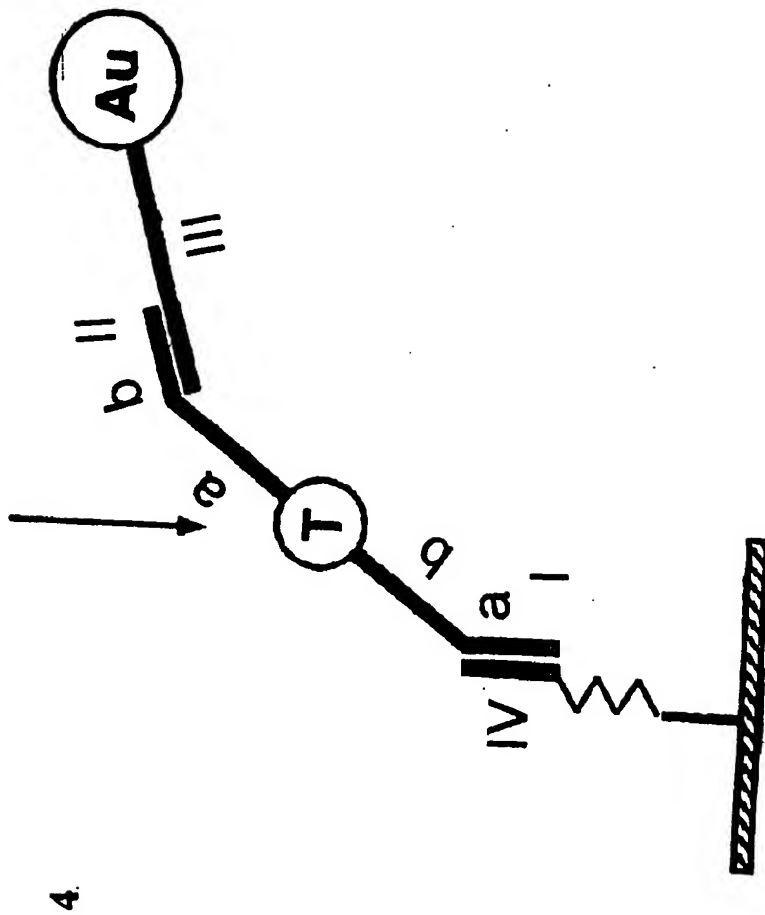
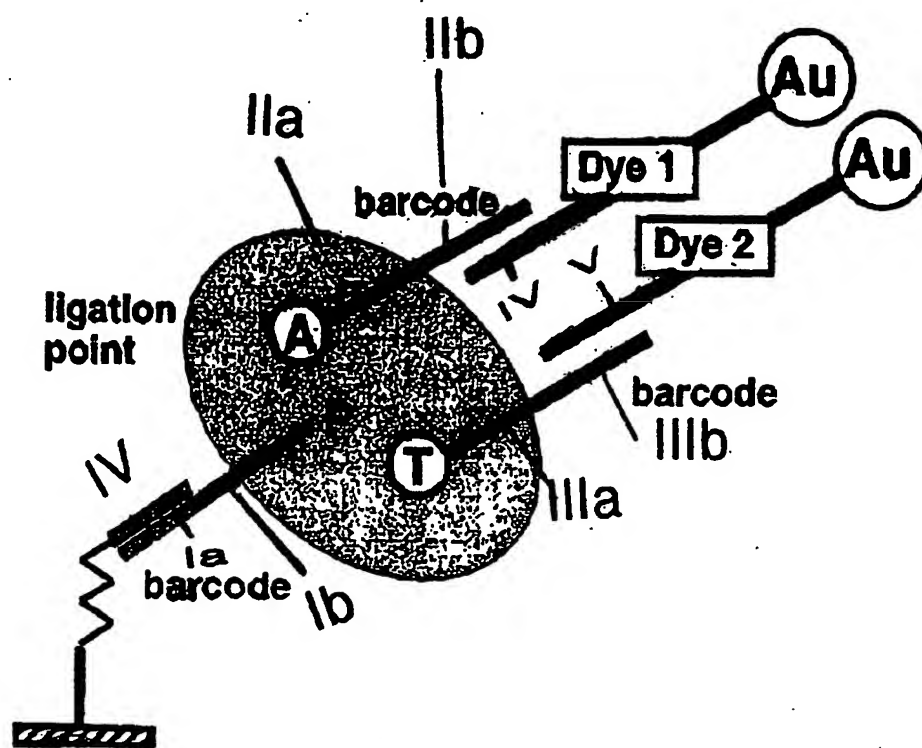


FIG. 4/5

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array capture (1 spot, 2 alleles)

FIG. 5/5